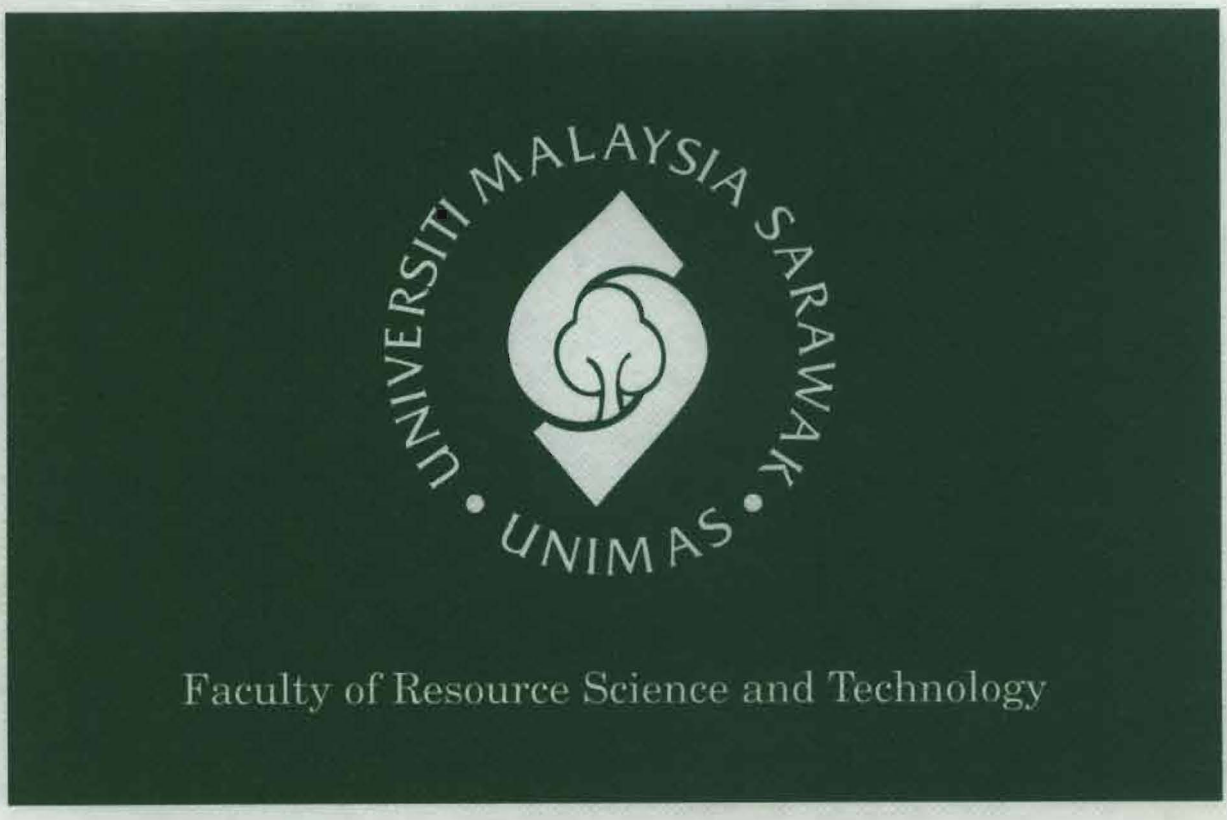


CYANOBACTERIA : CELL ISOLATION TECHNIQUE AND ESTABLISHMENT OF CLONAL CULTURES

Mardhiah Binti Abdul Majid

Bachelor of Science with Honours
(Aquatic Resource Science and Management)
2006

CYANOBACTERIA: CELL ISOLATION TECHNIQUE AND ESTABLISHMENT OF CLONAL CULTURE

MARDHIAH BINTI ABDUL MAJID

This project is submitted in partial fulfillment of the requirements for the degree of
Bachelor of Science with Honours
(Aquatic Resources)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2006

ACKNOWLEGDEMENT

Alhamdulillah, fullest gratitude to the Almighty with His blessing, I was being able to complete my Final Year Project entitle Cyanobacteria: Cell isolation technique and establishment of clonal culture. I would like to express a deep thankful to my supervisor, Dr Ruhana Hassan, for her valuable guidance, encouragement and understanding throughout the whole my Final Year Project. I am so grateful to Mr Samsur B. Mohamad, my co-supervisor for his advices and encouragement during this work as well as to other lecturer for their indispensable advices. Thanks also to Mr Stephen and Mr David from Tarat Inland Fisheries Division, for his permission to do sampling at fish pond in Tarat. I also would like to thank to the all aquatic lab assistant for their collaboration and assistance for me to complete this project. Finally, I am deeply indebted to all my friends for their help and constructive criticism and last but not least, my beloved parents for their fully support and encouragement throughout my project completion.

TABLE OF CONTENT

Cyanobacteria: Cell isolation technique and establishment of clonal culture

Mardhiah Abd Majid

Aquatic Resource Science and Management
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

A study was carried out to identify and isolate selected cyanobacteria species, found to be predominant causing blooms on the water surface in freshwater environment. The water samples were collected from three different fish ponds in Indigenous Fisheries Research and Production Centre, Tarat Inland Fisheries Division, Serian. Three species were identified from the collected samples, namely *Microcystis* sp., *Oscillatoria* sp. and *Anabaena* sp. These cyanobacteria were potentially cyanobacterial toxin producers. Cell isolation was carried out by streaking method procedure and consequently establishes the liquid culture of the small volume. The cultures were incubated in BG11 and f/2 medium for 25° C with 7000 lux light intensity (12:12, light: dark). *Microcystis* sp. was successfully isolated and cultivated. However, the *Oscillatoria* sp. and *Anabaena* sp. clonal cultures were faced difficulties to establish. This study also found that different morphology namely unicellular, colonial and filamentous required different cell isolation protocol and medium composition. The best amount of initial number cell density to successfully clonal cultures the *Microcystis* sp. was between 1.00 x 10^4 cells/ ml to 1.50 x 10^4 cells/ ml.

Keywords: cyanobacteria, isolation, BG11, f/2, fish pond

ABSTRAK

Satu kajian mengenalpasti dan memencilkan spesis alga biru-hijau yang diketahui sebagai punca utama ledakan alga biru-hijau yang sering berlaku di permukaan persekitaran air tawar telah dijalankan. Sampel air diambil daripada tiga kolam ikan yang berlainan di Pusat Penyelidikan dan Pengeluaran Perikanan Sungai, Tarat, Serian. Tiga spesies alga biru-hijau telah dikenalpasti daripada sampel air yang diambil iaitu Microcystis sp., *Oscillatoria* sp. *dan Anabaena* sp. *Ketiga-tiga spesis ini merupakan alga biru-hijau yang berpotensi untuk menghasilkan toksin. Teknik yang terlibat dalam proses pemencilan ialah kaedah 'streak plate' dan seterusnya menghasilkan pengkulturan dalam isipadu yang kecil. Kultur telah diletakkan dalam medium BG11 dan f/2 dengan suhu 25° C dan 7000 lux keamatan cahaya (12:12, terang: gelap). Microcystis* sp. *telah berjaya dipencilkan dan dikulturkan tetapi Oscillatoria* sp. *dan Anabaena* sp. *tidak berjaya dipencilkan. Hasil eksperimen mendapati alga biru-hijau yang mempunyai morfologi yang berbeza iaitu unisel,koloni dan berfilamen memerlukan kaedah dan komposisi médium yang berbeza untuk berjaya dipencilkan. Jumlah kepadatan sel permulaan yang sesuai dalam menghasilkan pengkulturan Microcystis* sp. *dalam densiti yang kecil ádalah antara 1.00 x 10^4 cells/ ml hingga 1.50 x 10^4 cells/ ml.*

Kata kunci: alga biru-hijau, pemencilan, BG11, f/2, kolam ikan

1.0 INTRODUCTION

The cyanobacteria are included in Kingdom Monera and under Cyanophyta Division (Figure 1.1) (Bold and Wynne, 1985). They consist of three orders, which are Chrooccocales, Oscillatoriales and Chaemasiphonales, and the most common found cyanobacteria are the order Chrooccocales. This systematic referred as the differentiation of cyanobacteria based on the difference of their morphology structure.

According to Bold and Wynne (1985), cyanobacteria can be classified into algae because they contain chlorophyll *a* which differs from the chlorophyll of the photosynthetic bacteria while based on Stanier *et al.* (1971), cyanobacteria are the bacteria because of their cellular organization and biochemistry. However, based on Graham and Wilcox (2000), cyanobacteria are basically known as the blue-green algae. Thus, the designation blue-green algae are therefore misleading, although this common name is now so firmly established.

About 150 genera and 2000 species of cyanobacteria can be found at the most diverse habitat (Hoek *et al.*, 1995). They can be found from freshwater to the marine habitat and either planktonic or benthic organisms. Their special features which are the akinetes and heterocysts allow them to survive in wide range of surroundings. Many species are capable of living in the soil and other terrestrial habitat where they are important in the functional process of ecosystems and the cycling of nutrient elements (Whitton, 1992). Furthermore, according to Humm and Wicks (1980), most marine

species forms grow along the shore as benthic vegetation in the zone between the high and low tide marks and according to Hoek *et al.* (1995), blue-green algae occur in marine, freshwater and terrestrial habitat. As the matter of fact, cyanobacteria can be found in many different habitat or ecosystem around the world.

The significance study on cyanobacteria is mainly due to the public health concern as well as the mortality of aquatic organisms reported worldwide. Nowadays, cyanobacterial blooms have been detected in freshwater ponds and lakes all over the world. Moreover, cyanobacteria blooms have several consequences for water quality and their collapse frequency causes high mortality among aquatic population (Vasconcelos *et al.*, 2001). Some of the cyanobacteria were found to be the major source of some potent biotoxins (Carmichael, 1998). Some species for instance the *Microcystis* sp. can produce toxin which is microcystin-LR. This toxin is often mentioned as the most frequently occurring microcystin (Sivonen and Jones, 1999).

As the matter of fact, the research about cyanobacteria is very important to determine the cyanobacteria species that can develop toxins during blooming events. According to Chorus and Bartram (1998), among cyanobacteria species that can develop toxins blooms, *Microcystis aeruginosa* is the most common and is a matter of great concern due to the properties of biotoxin that is highly potent.

The isolation technique of cyanobacteria has been developed by using medium to isolate and purify the cyanobacteria in order to determine the properties of the toxin.

Instead of that, the clonal cultures of single cell species also can provide the DNA information among the species in order to enhance species differentiation of molecular basis. Normally used medium of isolating cyanobacteria are BG11 (Allen, 1968), BG13 (Ferris and Hirsch, 1991) and Z8 (Rippka, 1988), depending on their species and morphological structure.

1.1 Objective

1.1.1 To identify the cyanobacteria species and establish the method of isolation and purification of cyanobacteria.

1.1.2 To establish clonal culture method of the cyanobacteria in laboratory conditions.

Kingdom: Monera

Division: Cyanophyta

Order: Chroococcales

Family: Chroococcaceae

Order: Oscillatoriales

Family: Oscillatoriaceae

Nostoceceae

Scytonematoceae

Stigonematoceae

Rivulariaceae

Order: Chaemasiphonales

Family: Pleurocapsaceae

Dermocarpaceae

Chaemasiphonaceae

Figure 1.1: Systematic of cyanobacteria taxon according to Bold and Wynne (1985).

2.0 LITERATURE REVIEW

2.1 Cyanobacteria

Cyanobacteria are found everywhere in the world ecosystem. Cyanobacteria have an impressive ability to colonize infertile substrates such as volcanic ash, desert sand and rocks and they can be found in extremely high or low habitat such as hot springs (Castenholz, 1973). Many species of cyanobacteria are capable of living in the soil and other terrestrial habitat, where they are important in the functional processes of ecosystems and the cycling of nutrient element (Whitton, 1992). They are the primary producers in the ecosystem which represent the higher trophic level of food chain and play an important role in providing the direct nitrogen to the environment, through nitrogen fixation process (Mur *et al.*, 1999). In spite of great importance of cyanobacteria for aquatic life nutrition, especially for fish growth and productivity, many of them were recorded to be highly toxic affecting the growth of aquatic organisms (Turrell and Middlebrook, 1988).

Cyanobacterial blooms have become an increasing problem worldwide in both freshwater and marine aquatic environment (Fastner *et al.*, 2003). According to Sivonen and Jones (1999), it seems likely that every country of the world have water bodies which support blooms of toxic cyanobacteria at sometimes or another and at least 46 species have been shown to cause toxic in invertebrate. Lagos *et al.* (1999) have reported, during the screening of toxic freshwater cyanobacteria in Brazil, three strains from

Cylindrospermopsis raciborskii species isolated from the state of Sao Paulo were found toxic by the mouse bioassay. The extracts of cultured cells caused acute death to mice due to its neurotoxin. Jose Maria *et al.* (2005) found that toxic cyanobacteria in public water supply reservoirs represent a serious health risk as they can release potent cyanotoxins into the water. The cyanobacterial biota included potentially toxic genera such as *Aphanizomenon, Microcystis, Nostoc, Oscillatoria, Planktothrix* and *Radiocystis*. Based on Carmichael *et al.* (1998), most of the toxic cyanobacteria genera have been recognized to produce a range of hepatotoxic toxins known as microcystins. Among the toxic cyanobacteria, *Microcystis* is the common and cosmopolitan genus from which 35 variants of microcystins have been isolated (Sivonen and Jones, 1999).

Although the freshwater cyanobacteria blooms are known to have worldwide occurrence, hence there are less study of cyanobacteria that undergo in Asian region. In Malaysia, there are some studies on cyanobacteria and those are from Sarawak (Abang Mohammad Zain, 2003 and Ramlah, 2005). According to a research on distribution of blue-green algae in selected environment in Sarawak (Abang Mohammad Zain, 2003), there are only three genera found in Sungai Sarawak which are from *Aphanocapsa, Aphanotheca* and *Chroococcus* while *Oscillatoria* is the common genera that found mostly in the freshwater environment for example Semariang Pond and Pengkalan Kuap River. Ramlah (2005) has reported, in lentic zone, there were different species compositions of cyanobacteria found such as *Anabaena* sp., *Microcystis* sp., *Lyngbya* sp., *Oscillatoria* sp., *Anacystis* sp., *Spirulina* sp., *Calothrix* sp., *Gleotrichia* sp. and *Chaemaesiphonales* sp.

2.2 Reproduction

Cyanobacteria can only reproduce asexually (Hoek *et al.*, 1995) and there are three types of reproduction form which are binary fission, spores and vegetative cell. The first reproduction form is the binary fission. According to Sze (1993), the division process occurs by the ingrowths of the cell membrane and wall at the equator of a cell and resulting in the formation of the new individual. On the other hand, few species of cyanobacteria can be reproducing by forming spores. Exospores result from successive division at one end of a cell while endospores are formed by multiple divisions within a cell and then are released when its wall splits open. The last reproduction form is through vegetative cell in filamentous cyanobacteria when the new cells are added to an existing filament as part of vegetative growth due to the fragmentation process.

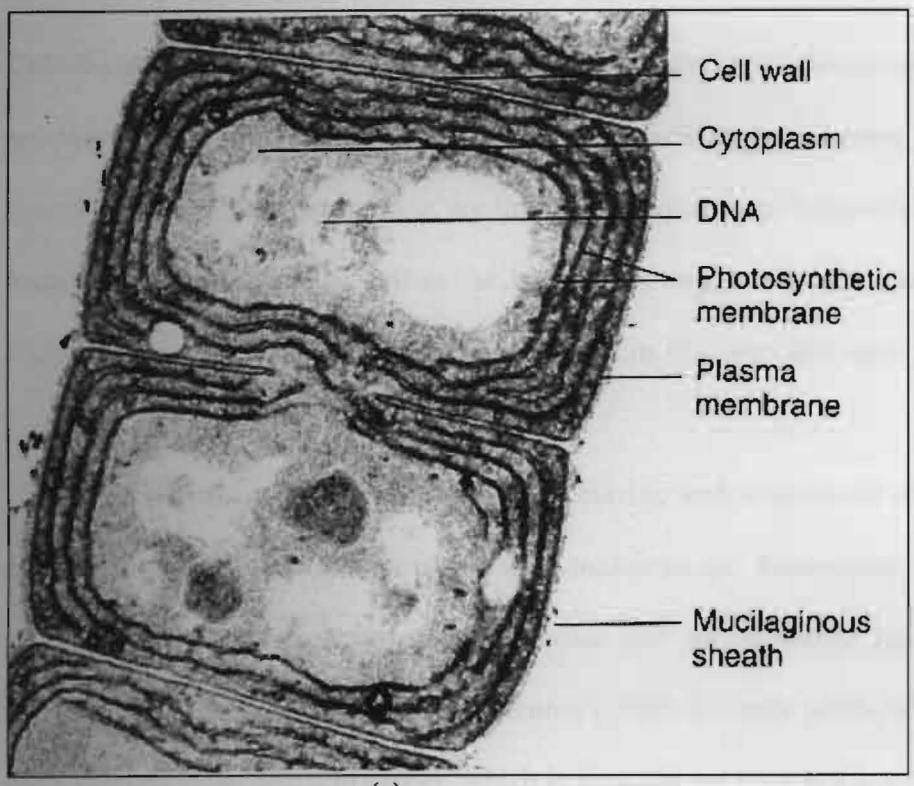
2.3 Structure and function

Cyanobacteria are prokaryotic algae that contain chlorophyll *a* in their thylakoids. The thylakoids contains only chlorophyll *a* while chlorophyll *b* and *c* are absent. As a prokaryotic alga, cyanobacteria possess a wall structure but do not possess membrane-bound sub-cellular organelles (Fay and Van Baalen, 1987 and Bryant, 1994). They also do not possess any organelles such as mitochondria, nucleus, golgi apparatus, endoplasmic reticulum and vacuoles and can be in the form of unicellular, colonial, filamentous and simple parenchymatous organization (Hoek *et al.*, 1995). Furthermore, the cells are generally blue-green to violet, but sometimes are red or green. The green of

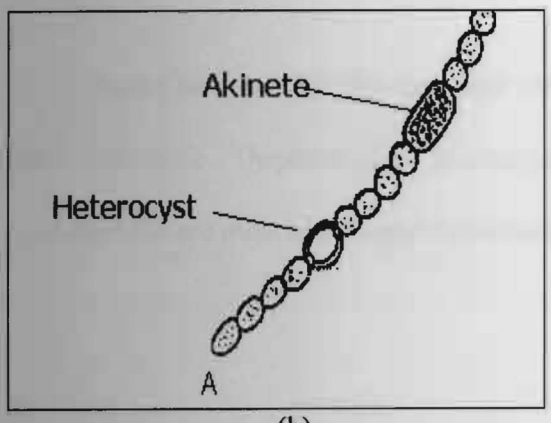
the chlorophyll is masked by the blue accessory pigment phycocyanin and allophycocyanin, and the red accessory pigment, phycoerythrin. Because of various proportions of these pigments, only about half of cyanobacteria are actually blue-green in color while many other species range in color from brown to olive green. Many species of cyanobacteria possess gas vesicles to regulate buoyancy and this is important for them to adjust their vertical position in the water column (Walsby, 1987).

2.4 Special features

Cyanobacteria can undergo nitrogen fixation process which is a fundamental metabolic process of cyanobacteria, giving them the simplest nutritional requirements of all living organisms. They use nitrogenase enzyme to convert directly nitrogen to ammonium that can be absorb through the food chain. Cyanobacteria with heterocycts (Figure 2.1 (a)) can keep their nitrogen requirements and grow even though the nitrate and ammonium level relatively low in the water (Hoek *et al.*, 1995). Nitrogen fixation cyanobacteria are among genera *Anabaena* and *Nostoc* (Stewart, 1973). While some cyanobacteria also produce spores which called akinetes (Figure 2.1 (b)) that are resistant to drying. These spores allow cyanobacteria to survive unfavorable environmental conditions.



(a)



(b)

Figure 2.1: Typical cyanobacteria cell (a), heterocyst and akinete (b).

2.5 Public concern in cyanobacteria study

The common toxic cyanobacteria in freshwater ecosystem are *Microcystis* sp., *Cylindrospermopsis raciborskii*, *Plankthorix rubescens*, *Synechococcus* sp., *Anabaena* sp., *Nostoc* sp., and *Oscillatoria* sp. *(Ocsillatoria agardhii)*. For example, *Microcystis* sp., commonly *Microcystis aeruginosa*, are link most frequently to hepatotoxic (microcystins toxin) blooms worldwide as well as the *Microcystis viridis* and *Microcystis botrys* strain which also have been shown to produce microcystins (Sivonen and Jones, 1999).

Microcystins are the most frequent occurring and widespread of the cyanotoxin and have been characterized for planktonic *Anabaena* sp., *Microcystis* sp., *Ocsillatoria* sp., *Nostoc* sp. and *Anabaenopsis* sp. species and for terrestrial Halosiphon genera (Sivonen and Jones, 1999). Based on Falconer (1993) the main pathway for microcystin entry into cell is the bile acid carrier, which is found in the liver cell and to a lesser extent in the intestinal epithalia.

Based on report by Sivonen and Jones (1999), throughout the world, it appears that liver-toxic (hepatotoxic, microcystin- containing) freshwater blooms of cyanobacteria are most commonly found than neurotoxin blooms.

Examples of several cases of toxin cyanobacteria in lakes and reservoir ecosystem:

1. First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German Lakes (Pseußel *et al.*, 2006).

2. Cylindrospermopsin occurrence in two German lakes and preliminary assessment of toxicity and toxin production of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates (Fastner *et al.*, 2003).

3. PSP toxins from *Aphanizomenon flos-aquae* (Cyanobacteria) collected in the Crestuma-Lever reservoir (Douro river, northern Portugal) (Ferreira *et al.*, 2001).

4. The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil (Lagos *et al.*, 1999).

3.0 MATERIALS AND METHODS

This section will cover seven materials and methods procedure which are the sampling site, water sampling, medium preparation, identification of species, cell isolation protocol, clonal cultures and data analysis, respectively.

3.1 Sampling site

The water samples were collected from 3 different ponds AP 11, NNPA 46, SEP 3 at Indigenous Fisheries Research and Production Centre, Tarat Inland Fisheries Division, Serian, and referred as Pond 1 (P1), Pond 2 (P2) and Pond 3 (P3), respectively.

During the first sampling, visible green bloom (green water) were seen in all ponds. The ponds were used for fish cultured and maintain under different purposes. P1 was for single species fish cultures which were the Empurau juveniles, P2 was for mixtures fish culture of Semah (*Tor duorenensis*), Mata Merah (*Osteochilus melanopleura*) and Mengalan (*Puntius bulu*) species and P3 was for brood stock fish pond.

3.2 Water sampling

Water samplings have been carried out twice in December 2005 and February 2006, at the same fish pond. Water samples were collected using bottle (1.0 L) at surface

water from P1, P2 and P3. The water samples were sieved through 20 μm mesh size sieve and were collected into a 250 ml beaker. All water samples were kept inside an incubator (Shel Lab) at 25°C (Rippka, 1988) before cyanobacteria cell isolation technique and establishment of clonal culture been undergone.

3.3 Medium preparation

Two types of medium were prepared (liquid and agar medium) which were used for isolating the cyanobacteria species and consequently for its clonal cultures establishment.

3.3.1 Liquid medium

Ingredients of original medium for BG11 (Allen, 1968) (Table 3.1) and modified f/2 medium (Guillard, 1975) (Table 3.2) were added accordingly into 1.0 L conical flask and mixed well. The mixed solution was then autoclaved at 120°C for 20 minutes and later placed inside the incubator after it was cooled to room temperature. The f/2 medium used in this project was the modified version by replacing the seawater component with distilled water and omitting the silica to allow cyanobacteria growth.

3.3.2 Agar medium

Concentration of agar for BG11 (Allen, 1968) and modified f/2 medium (Guillard, 1975) used in this project was one percent. For preparation of this solid media, 10.0 g of

agar (Agarose-Biotechnology Grade, BST Technology Lab) were added into the 1.0 L liquid media and mixed well. This solution was autoclaved at 120°C for 20 minutes. The autoclaved solution was then poured into sterile petri dish and allowed to cool to room temperature. Parafilm was used to seal the petri dish to avoid contamination.

Tables 3.1 and 3.2 below summarized materials needed for preparing the BG11 and f/2 medium.

Table 3.1: Composition of BG 11 medium for cyanobacteria (adopted from Allen, 1968).

Ingredient	Concentration	
	g/ L	mM
$NaNO_3$	1.500	17.65
K_2HPO_4	0.040	0.180
$MgSO_4.7H_2O$	0.075	0.300
$CaCl_2.2H_2O$	0.036	0.25
Citric acid	0.006	0.03
Ferric ammonium citrate	0.006	0.03
EDTA (disodium salt)	0.001	0.003
Na_2CO_3	0.020	0.19
Agar (if needed)	10.00	
Trace metal*		
Distilled water#		

*Trace metal mix (1.0 ml): H_3BO_3, 2.86 g: $MnCl_2.4H_2O$, 1.81 g: $ZnSO_4.7H_2O$, 0.222 g: $NaMoO_4.2H_2O$, 0.39 g: $CuSO_4.5H_2O$, 0.079 g: $Co(NO_3)_2.6H_2O$, 0.0494 g.

#Distilled water: 1.0 L

pH 7.1-7.5 after autoclaving and cooling

Table 3.2: Composition of f/2 vitamin solution (modified from Guillard, 1975).

Ingredient	Concentration	
	g/ L	mM
$NaNO_3$*	75.0	1.0 ml
$NaH_2PO_4.H_2O$*	5.0	1.0 ml
Trace metal solution*		1.0 ml
Vitamin solution*		1.0 ml
Distilled water		1.0 L

*$NaNO_3$: Distilled water, 1.0 L

*$NaH_2PO_4.H_2O$: Distilled water, 1.0 L

*Trace metal mix: $FeCl_3.6H_2O$, 3.25 g: $Na_2EDTA.2H_2O$, 4.36 g: $CuSO_4.5H_2O$, 1.0 ml: $Na_2MoO_4.2H_2O$, 1.0 ml: $ZnSO_4.7H_2O$, 1.0 ml: $CoCl_2.6H_2O$, 1.0 ml: $MnCl_2.4H_2O$, 1.0 ml: Distilled water, 1.0 L

* Vitamin solution: Vitamin B12, 1.0 ml: Biotin, 1.0 ml: Thiamine, 200.0 mg: Distilled water, 1.0 L

3.4 Identification of species

One ml of water samples was pipetted into a 12 multi well plate and examined under Olympus Inverted Microscope (1 X 51- RFL- (a)). Detail examination of the sample was carried out to determine the occurrence of unicellular, unicolony or unifilament cyanobacteria. Photographs were captured and kept as records. Samples were identified based on Anagnostidis and Komarek (1985), (1988) and (1991), Komarek and Anagnostidis (1986) and (1989), Prescott (1970), Aisyah (1996) and Falconer (1993). After the presence of cyanobacteria in the water samples was confirmed, the screening procedure was carried out accordingly.

3.5 Cell isolation protocol

Cell isolation protocol was carried out by two main procedures which were the screening procedure (Rippka, 1988 and Thiery *et al.*, 1991) and isolation and purification method (Stanier *et al.*, 1971).

3.5.1 Screening procedure

Two steps of screening procedure were carried out: (i) the agar medium screening and (ii) liquid medium screening. Both procedures were undergone to determine the existence of cyanobacteria from the water samples.

3.5.1.1 Agar medium screening

Method applied in this project was according to Rippka (1988) and Thiery *et al.* (1991). One ml of water samples was streaked directly onto plates of BG11 and f/2 agar medium and seal with Parafilm. The petri dishes were incubated at 25°C under 94.5 μEin m^{-2} s^{-1} cool white fluorescent intensity with 12:12 (light: dark) photoperiod in the incubator. The appearance of green colonies on the dish showed the growth of cyanobacteria cell.

3.5.1.2 Liquid medium screening

One ml of water samples was transferred into 100 ml conical flask containing BG11 and f/2 liquid medium to enrich the cyanobacterial population prior to plating. For the filamentous cyanobacteria, dilution procedures were used to dilute the freshwater samples in order to easily gain the single individual. One ml of the water samples (enrich liquid medium) was diluted into 9 ml of liquid medium of BG11 and f/2 in a test tube. The visibility of green bloom in the test tube showed the growth of cyanobacteria cell. Test tubes were incubated at 25°C under 94.5 μEin m^{-2} s^{-1} cool white fluorescent intensity with 12:12 (light: dark) photoperiod in the incubator.

3.5.2 Isolation and purification method

This method was adapted from Stanier *et al.* (1971). After the green colonies on the dish appeared (in section 3.5.1.1), a sterile wire loop was used to inoculate the colony to other new petri dish. Restreaking method was applied to gain unicellular cyanobacterium after the screening process. On the other hand, for unifilament species, the diluted water samples (as in section 3.5.1.2) were transferred into 12 multi well plate cell culture and few transferring procedure were done to obtain a single individual cell. The plates were incubated at same condition as referred earlier. All the isolation technique procedure was done under laminar flow hood (ERLA CFM Series).

3.6 Clonal cultures

A green colony from petri dish in section 3.5.2 above was inoculated into 100 ml fresh BG11 medium in 3 different Erlenmeyer flask by using sterile wire loop under laminar flow hood. After a week, 1.0 ml of liquid culture from the initial stock culture flask was transferred into 3 new flasks with 100 ml fresh BG11 medium. Liquid cultures of 100 ml volume (including stock cultures) were grown without aeration at 25°C under 94.5 μEin m^{-2} s^{-1}cool white fluorescent light intensity with 12:12 (light: dark) photoperiod in the incubator. Three different initial cell densities were selected for this three experiments, which were 0.400 x 10^4 cells/ ml, 1.0 x 10^4 cells/ ml and 1.20 x 10^4 cells/ ml, respectively, in order to measure the optimum growth pattern of isolated species. Samples (10 μL) for cell counting were taken daily for a week and a

hemacytometer (Improved Neubauer) was used to count the number of cell in the flasks. Samples were previously shaken in order to break up the colonies for easy counting. Graph shows the growth curve of the sample were plotted and maximum cell density were determined. The formula used for cell counting using hemacytometer as follows (Guillard, 1973):

$$\frac{Y}{Z} \times 10^4$$

Y= number of individual cell counted on hemacytometer

Z= number of blocks

In this study, only five blocks were used for cell counting of the individual.

3.7 Data Analysis

The cell counting data have been plotted on cell density ($x10^4$ cells/ml) against day graph. The program used was the Microsoft Office Excel.